New PCT Application
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Our Ref.:   E 2545 PCT

Use of γ–GT inhibitors for the treatment of degenerative diseases

The present invention relates to the use of γ–GT inhibitors for the preparation of pharmaceutical compositions for the treatment of degenerative diseases. In particular it pertains to the treatment of chronic renal or inner ear conditions or injuries which are reactive oxygen species (ROS) induced.

Degenerative diseases are considered to be diseases which are linked to chronic disorders and/or chronic physiological damages in the human or animal body.
Besides degenerative diseases of, inter alia, the centrol nervous system, chronic disorders of the kidneys or the liver may lead to degeneration of the corresponding tissues. For example renal diseases, or inner ear degenerative diseases are frequent pathological conditions for which hardly any treatment is available.

In particular, glomerulosclerosis and other renal diseases are a frequent complication of many chronic conditions including diabetes (Ha and Kim, Diabetes Res. Clin. Pract. 45 (1999), 147-151). Thereby an excess of reactive oxygen species (ROS) is thought to play a crucial role (Haugen and Nath, Blood Purif. 17 (1999), 58-65). Animal models have been developed to study the pathomechenisms involved. In a genetic mouse model of otorenal disease (Meyer zum Gottesberge, European Archiv Otorhinolaryngology 253 (1996), 470-474; Weiher, Cell 62 (1990), 425-434) it has been shown that interventional therapy using radical scavengers is effective (Binder, Am. J. Pathol. 154 (1999), 1067-1075). However, antioxidant therapy in humans would be rather difficult to carry out with the desired specificity, considering, for instance, the particular oxygen species to be scavenged as well as its site of action. Therefore, it would be advantageous to inhibit specifically the enzymatic activity that generates the particular ROS responsible for the tissue damage.

If the prior art has proposed a role of ROS in glomerulosclerosis nothing was known about the actual source of the damaging enities in said disease. Recently, reaction conditions have been defined in vitro, in which ROS are formed as a consequence of the action of the enzyme γ–GT ((Drozdz et al., 1998)).

Although an important role of ROS in the pathological-mechanism of glomerulosclerosis has been recognized in the prior art, it was not known which of the many possible mechanisms to generate said oxygen species is responsible.

Therefore, the technical problem of the present invention was to provide for means and methods which may be used in the treatment and/or alleviation of degenerative, chronic diseases. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the present invention relates to the use of γ-GT inhibitors for the preparation of a pharmaceutical composition for the treatment of a degenerative disease.

In this invention it was surprisingly found that overexpression of the enzyme γGT is a source of damaging ROS in the kidney and other cells, in particular of cells of the inner ear. Thus, the inhibition of γGT (systemic or local) should lead to means and methods for successfully and effectively preventing the progress of the chonic tissue damage imposed by elevated ROS levels in kidney and inner ear.

Drozdz (Free Radical Biology and Medicine 25 (1998), 786-792) has speculated based on an in vitro experiment, that γ-GT may be involved in the generation of ROS. Yet, these investigations provoked an ROS generation in vitro by applying a glutathione/transferrin system to cultured V79 cells. These authors showed a rather fast ROS generation by γ-GT when glutathione and transferrin was present. This in vitro generation of ROS reached a maximal rate in the first six minutes. However, implications for longer and/or constant damage by ROS in an in vivo situation could not be drawn by these published data.

Furthermore, in this paper it was not addressed whether this mechanism had any relevance on the organismal level in vivo. There were no implications for a role of this in persistent damage by ROS leading to chronic tissue lesions.

Yet, in this invention it could be surprisingly shown (see appended examples) that excess γ–GT activity and increased extracellular ROS can be found in vivo. In addition, and in contrast to Drodz et al. (loc. cit., 1998), in this invention it could be demonstrated, that in a model of chronic disease the increased extracellular ROS, identified as causal for the disease, was accompanied by excess γGT activity. Furthermore, it was found that the membrane localised γ–GT enzyme, which commonly is described as a oxygen protective activity (by enhancing intracellular Cys-Gly levels, and therefore allowing for intracellular accumulation of glutathione), actually produces extracellular Cys-Gly concentrations involved in ROS formation and that excess γ–GT activity results in increased extracellular ROS in in vivo situations. It can therefore be reasoned, that the membrane localised γ–GT causes ROS-mediated chronic damages.

Therefore, the present invention provides for the above mentioned use of γ-GT inhibitors in degenerative chronic diseases.

γGT inhibitors and their medical use are known in the art. Inter alia, US 4,758,551 describes the use of such inhibitors for γ-GT in the protection of kidneys from metal or toxin poisoning. However, in US 4,757,551 it was speculated that γ-GT activity allows for the uptake and metabolism of these toxic substances. A link to degenerative and/or chronic diseases was neither shown nor speculated.

Degenerative diseases according to the invention are particularly chronic renal diseases or inner ear degenerative diseases or injuries. It is, as mentioned above, preferred that these diseases are chronic disorders and/or ROS-induced.

Particularly preferred examples of chronic renal diseases are focal and/or segmental glomerulosclerosis, minimal change nephrosis, inflammatory and/or autoimmune glomerulopathies. Yet, it is also envisaged that said chronic renal disease is diabetic nephropathy.

In the context of the invention, particularly preferred examples of inner ear injuries are sensineural deafness induced by age, physiological status, metabolical status and/or drugs. Exemplary examples for said drugs are aminoglycosides or cisplatin dervatives. A preferred example for an inner ear degenerative condition according to the invention is otosclerosis.

Particularly preferred γ–GT-inhibitors in context of this invention and which may be employed in the above described use are AT 125 (Acivicin) or its derivatives. In a preferred embodiment, peptide inhibitors of the CysGlyXtype may be employed, wherein X may either stand for any of the naturally occurring aminoacids or a modified aminoacid, an oligo- or a polypeptide may be used according to the invention. Further, peptide inhibitors are inhibitors γ-GluXYtype, wherein X and Y may either stand for any of the naturally occurring aminoacids or a modified aminoacid, an oligo- or a polypeptide may be used according to the invention. Furthermore, peptidomimetic glutathion analogues (Burg, Bioorg Med Chem 10 (2002),195-205), compounds or derivatives of the type L-2-amino-4-boronobutanoic acid (ABBA) (London and Gabel, Arch Biochem Biophys 385 (2001), 250-258), and anilides, such as γ-glutamyl-7-amido-4-methylcoumarin (γ-Glu-AMC) (Stein, Biochemistry 40 (2001), 5804-5811) may be used in accordance with this invention.

The pharmaceutical compositions according to the invention can be administered by any appropriate route such as oral, buccal, sublingual, parenteral, topical or by inhalation and can be formulated according to methods well known in the art. Accordingly, the present invention also provides for a method of treatment, prevention and/or amelioration of a degenerative disease, in particular of chronic renal diseases or inner ear degenerative conditions or inner ear injuries comprising the step of administering to a patient in need thereof a pharmaceutical composition as defined herein. Preferably said patient is a human patient.

Typically an effective amount of the effective compound will be admixed with a pharmaceutically acceptable carrier, optionally using further excipients known to the person skilled in the art if appropriate.

It will be appreciated by the person skilled in the art that the route of application and the daily dosis is dependent on individual parameters such as severity of the condition/disease treated, concommitant diseases and medication, age and body weight of the patient.

The Figures show:

Figure 1: ESR spectra from DMPO-OH adducts produced in fibroblasts.

A, B: Mpv17-/- (LUSVX) cells were reacted with DMPO for 60 min. B: Reaction as in A in the presence of 30 units/ml SOD. C: Mpv17 expressing (NIX15) cells treated as in A. D: Reference spectrum of the DMPO-OH adduct generated by reaction of DMPO with H_2O_2 upon UV radiation. Representative results from three independent experiments.

Figure 2: mRNA levels of fibroblasts in culture.

A: Ratio of mRNA levels in Mpv17-/- versus Mpv17 expressing cells. B: Acivicin dependent ratio of mRNA levels in Mpv17-/- cells. ß-actin mRNA was used for reference.

(Columns represent means ± S.D., n=3)

The following examples described below establish the usefulness of γ-GT inhibitors for the treatment of ROS induced chronic renal diseases and inner ear degenerative diseases and injuries.

Example 1: ROS-generation in Mpv17 mice and its experimental investigation

The Mpv17 mouse strain is a genetically recessive rodent model of glomerulosclerosis (H. Weiher, T. Noda, D. A. Gray, A. H. Sharpe and R. Jaenisch (1990), *Cell*, **62**, pp. 425-434) and sensineural deafness (A. M. Meyer zum Gottesberge, A. Reuter and H. Weiher (1996), *European Archiv Otorhinolaryngology*, **253**, pp 470-474). Kidney and inner ear show marked changes in the extracellular matrix composition of glomerular and cochlear basement membrane components

and of matrix metalloproteinase 2 (MMP-2) (A. Reuter, A. Nestl, R.M. Zwacka, J. Tuckermann, R. Waldherr, E. M. Wagner, M. Hoyhtya, A. M. Meyer zum Gottesberge, P. Angel and H. Weiher (1998), *Molecular Biology of the Cell*, **9**, pp. 1675-1682). The Mpv17 gene, which is insertionally inactivated in mutant mice, codes for a peroxisomal hydrophobic 20 kDa protein with yet unknown molecular function (R. M. Zwacka, A. Reuter, E. Pfaff, J. Moll, K. Gorgas, M. Karasawa and H Weiher (1994), *EMBO Journal* **13**, pp 5129-5134). A role of reactive oxygen species (ROS) in the pathogenesis was recently established, as oxygen radical scavengers attenuate the development and progression of the disease (C. J. Binder, H. Weiher, M. Exner and D. Kerjaschki (1999), *American Journal of Pathology*, 154, pp. 1067-1075).

In the experiments/examples described below ROS generated in Mpv17-/- cells were detected by electron spin resonance (ESR). Furthermore, activities and expression of enzymes of the antioxidant defense line were studied in Mpv17-/- kidneys and cells in culture. The results reveal an imbalance of the activity and/or gene expression of some protective enzymes. A novel route of γ-glutamyl transpeptidase (γ GT) in the development of oxidative damage is proposed in accordance with a pro-oxidant effect of up-regulated γ-glutamyl transpeptidase recently reported for short-term ischemia of rat kidney (Cutrin, Kidney International 57 (2000), 526-533). As in the Mpv17 animal model, γ-glutamyl-transpeptidase regulation may be important in the investigation of human kidney and inner ear disease.

Kidneys from background mice (CFW x Balb/c) and transgenic homozygous Mpv17-/- mice (H. Weiher, T. Noda, D. A. Gray, A. H. Sharpe and R. Jaenisch (1990), *Cell*, **62**, pp. 425-434) were obtained at an age of 7 to 9 months and frozen in liquid nitrogen. LUSVX cells (SV40 immortalised fibroblasts from the lungs of Mpv17-/- mice), and NIX15 cells (Mpv+/+ cells derived from LUSVX cells by stable transfection of an expression construct for the human Mpv17 cDNA) (A. Reuter. A. Nestl, R. M. Zwacka, J. Tuckermann, R. Waldherr, E. M. Wagner, M. Hoyhtya, A. M. Meyer zum Gottesberge, P. Angel and H. Weiher (1998), *Molecular Biology of the Cell*, **9**, pp. 1675-1682) were grown in DMEM including 10% fetal calf serum (FCS) and 1 µg/ml puromycin (NIX15 cultures) at 6.5 % CO_2 Cells were harvested by trypsinisation and each 5 x 10^6 cells were washed in 1.5 ml of phosphate-buffered saline (PBS) before

freezing the pellet in liquid nitrogen. Where indicated, Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP), final concentration 10 μM (Y. Noda, M. Kohno, A. Mori and L. Packer (1999), *Methods in Enzymology*, **299**, pp. 28-34) (Calbiochem, Schwalbach, FRG), or the γ–GT inhibitor acivicin (AT-125), final concentration 50 μM (Sigma, Deisenhofen, FRG) were added in DMEM/10% FCS 18 h before harvesting the cells. Kidneys and cells were stored frozen at -80°C.

Electron spin resonance (ESR) measurements were carried out as follows:
Radical production in lung fibroblasts from Mpv17-/- animals (LUSVX) was identified by ESR and compared to Mpv17 expressing fibroblasts (NIX15). For ESR determination of generated ROS (K. M. Faulkner, S. I. Liochev and I. Fridovich (1994), *Journal Biological Chemistry*, 269, pp. 23471-23476), 5,5 dimethyl-1-pyrroline N-oxide (DMPO) (Sigma, Deisenhofen, FRG) was used as spin trap. To 1 ml of cell suspension (1.5×10^6/ml in PBS) a final concentration of 6.5 mM DMPO (kept under nitrogen at -20°C after charcoal filtration) was added. After 1 h at 37°C (for the samples represented in Fig.1 C below in the presence of 30 U/ml Superoxide dismutase) the samples were stored in liquid nitrogen. ESR was performed on a Bruker ESP300 instrument. The settings were: modulation amplitude: 2 G; gain: 1.6×10^6, power: 40mW, sweep 100G/40s = 2.5 G/s. A reference spectrum was derived by reaction of DMPO with H_2O_2 upon UV radiation.

Enzyme assays and glutathione assays were carried out as follows:
Cell fractions were prepared by differential centrifugation from 20% tissue and cell homogenates (w/v) in 10 mM potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose, 150 mM potassium chloride, 1 mM EDTA, 2 mM dithiothreitol, and 50 μM phenylmethylsulfonyl fluoride. Enzyme activities were determined at 25°C: catalase in the 25,000 x g (10 min) pellet H. E. Aebi (1983), In *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer), Verlag Chemie, Weinheim, pp. 273-276); glutathione peroxidase (GPx) (R. A. Lawrence and R. F. Burk (1976), *Biochemical Biophysical Research Communications*, **71**, pp. 952-958), GSSG reductase (Goldberg, D. M. S. R. F. (1983) In *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer) Verlag Chemie, Weinheim, pp. 258-265), glutathione transferase (GST) with 1-chloro-2,4-

dinitrobenzene (CDNB) W. H. Habig, M. J. Pabst and W. B. Jakoby (1974), *Journal Biological Chemistry*, **249**, pp. 7130-7139, and superoxide dismutase (SOD) (S. Marklund and G. Marklund (1974), *European Journal of Biochemistry*, **47**, pp. 469-474) in the 105,000 x g (45 min) supernatant; γ-glutamyl transpeptidase (γ-GT)) (S. Marklund and G. Marklund (1974), *European Journal of Biochemistry*, **47**, pp. 469-474) in the resuspended 105,000 x g pellet containing 1% Triton X-100. Protein was measured by the method of Bradford using bovine serum albumin as standard (M. M. Bradford (1976), *Analytical Biochemistry*, **72**, pp. 248-254).

Glutathione equivalents (GSH + 2 GSSG) were assayed in kidney cytosols following the formation of 5-thio-2-nitrobenzoate (TNB) spectrophotometrically at 405 nm (T. P. Akerboom and H. Sies (1981), *Methods in Enzymology*, **77**, pp. 373-382)

mRNA levels were also measured. In particular, levels of mRNA were determined by quantitative RT-PCR. Five µg of total RNA (F. M. Ausubel (1995) *Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology*. Wiley Press, New York.) were used in each cDNA synthesis reaction (Superscript II reverse transcriptase system, Gibco). Primers were selected from published sequences: mouse γ–GT (Z. Z. Shi, G. M. Habib, R. M. Lebovitz and M. W. Lieberman (1995), *Gene*, **167**, pp. 233-237), mouse cellular glutathione peroxidase (cGPx) (I. Chambers, J. Frampton, P. Goldfarb, N. Affara, W. McBain and P. R. Harrison (1986), *EMBO Journal*, **5**, pp. 1221-1227), mouse plasma glutathione peroxidase (pGPx) (R. L. Maser, B. S. Magenheimer and J. P. Calvet (1994), *Journal of Biological Chemistry*, **269**, pp. 27066-27073), mouse non-selenium glutathione peroxidase (nsGPx) B. Munz, S. Frank, G. Hubner, E. Olsen and S. Werner (1997), *Biochemical Journal*, **326**, pp. 579-585), mouse phospholipid hydroperoxide glutathione peroxidase (PHGPx) (S. Nam, N. Nakamuta, M. Kurohmaru and Y. Hayashi (1997), *Gene,* **198**, pp. 245-249), mouse glutathione reductase (glu red) (M. Tutic, X. A. Lu, R. H. Schirmer and D. Werner (1990), *European Journal of Biochemistry*, **188**, pp. 523-528), mouse copper-zinc superoxide dismutase (CuZnSOD) (G. C. Bewley (1988), *Nucleic Acids Research,* **16**, p. 2728), mouse manganese superoxide dismutase (MnSOD) (R. A. Hallewell, G. T. Mullenbach, M. M. Stempien and G. I. Bell (1986), *Nucleic Acids Research*, **14**, p. 9539), mouse

extracellular superoxide dismutase (ecSOD) (J. G. Suh, S. Takai, T. Yamanishi, T. Kikuchi, R. J. Folz, K. Tanaka, Y. S. Oh and K. Wada (1997), *Molecules and Cells*, **7**, pp. 204-207) and mouse xanthine oxidase (XO) (M. Terao, G. Cazzaniga. P. Ghezzi, M. Bianchi, F. Falciani, P. Perani and E. Garattini (1992), *Biochemical Journal*, **283**, pp. 863-870). Reactions were standardized using rat β–actin as a control. (U. Nudel, R. Zakut, M. Shani, S. Neumann, Z. Levy and D. Yaffe (1983) *Nucleic Acids Research*, **11**, pp. 1759-1771). For each primer pair the cycle number was established in which the accumulation of reaction products was linear.

gene	primer sequences 5´-3´	no. of cycles	annealing temp. (°C)
γ–GT	GCTGTCCCTGGTGAAATCCG		
	GCATAGGCAAACCGAAAGGC	36-40	56
cGPx	GGGGCAAGGTGCTGCTCATT		
	GTACGAAAGCGGCGGCTGTA	26-30	59
pGPx	CGAGTATGGAGCCCTCACCA		
	CCAGCGGATGTCATGGATCT	34-40	58
nsGPx	GCTTCCACGATTTCCTGGGA		
	TGTTTGGCTTCCTCTTCGGA	26-30	56
plGPx	TCTGGCAGGCACCATGTGTG		
	ATCACCTGGGGCTCCTCCAT	22-26	59
glu red	AATTCAGTTGGCATGTCATCAAGCA		
	CTGTGTGAACTTCAACACCTCCACG	28-34	59
CuZnSOD	TGGCGATGAAAGCGGTGTGC		
	GCGGCTCCCAGCATTTCCAG	22-24	59
MnSOD	AACAACCTCAACGCCACCGA		
	CAATCCCCAGCAGCGGAATA	28-30	59
ecSOD	CGGCCTGTGGCTCTGTCACCATGT		
	CACCACGAAGTTGCCAAAGTCGCC	28-32	59
XO	CCTGCTTGACCCCCATCTGC		
	CGGACTTGACCTGCTTGCCA	30-32	58
β–actin	TCATAGATGGGCACAGTGTG		

CTAAGGCCAACCGTGAAAAG   22-26   59

Reactions were performed for 30 s at the annealing temperature, 40 s at 72°C for synthesis and 30 s at 95°C for denaturation. Amplified mRNA was separated on 1.5 % agarose gels in Tris borate buffer (F. M. Ausubel (1995) *Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology*. Wiley Press, New York) visualized with vista green (Amersham, Freiburg, FRG) and quantified with a FLA2000 Imager (FUJI).

Example 2: Mpv17 expression diminishes superoxide production in fibroblasts from Mpv17 -/- mice

Spin-adducts formed in the presence of Mpv17-/- cells (LUSVX) showed a profile as depicted in Figure 1A, thus characteristic of a profile of DMPO-OH adducts (compare to Figure 1D). Adducts were found mostly in the supernatant rather than within the cells (not shown) indicating that they were either formed within the cell and secreted, or generated by ROS released into the medium. When the reaction was performed in the presence of exogenous superoxide dismutase (SOD) the DMPO-OH peak decreased to about one-third (Figure 1B) indicating that most of the stable DMPO-OH adducts were generated indirectly via an ·OOH-adduct formed by the reaction with superoxide. With Mpv17 expressing NIX 15 cells, generated by transfection of Mpv17-/-cells (LUSVX), the concentration of DMPO-OH was decreased by about 35% as compared to Mpv17-/- cells (LUSVX) (Figure 1C).

Example 3: Changes in the glutathione cycle in kidneys of Mpv17-/- mice

Since cultured LUSVX cells (Mpv17-/-) produce elevated levels of superoxide and oxidative damage has been shown to be causal to the development of the disease in Mpv17-/- mice (C. J. Binder, H. Weiher, M. Exner and D. Kerjaschki (1999), *American Journal of Pathology*, **154**, pp. 1067-1075), enzymatic activities relevant to superoxide production were determined in kidneys of Mpv17-/- mice showing progressed glomerulosclerosis at an age of 7-9 months (C. J. Binder, H. Weiher, M. Exner and D. Kerjaschki (1999), *American Journal of Pathology*, **154**, pp. 1067-1075)

(Table 1). As compared to the corresponding Mpv17+/+ kidneys, no significant difference in glutathione levels and in the activities of superoxide dismutase (SOD), catalase, GSSG reductase and glutathione transferase (GST) was observed in the kidneys of both mouse strains. However, in kidneys of Mpv17-/- mice γ-glutamyl transpeptidase (γ–GT) activity was increased by about two-fold, whereas glutathione peroxidase (GPx) activity was decreased by about one-third as compared to Mpv17+/+ animals. The following table illustrates the results:

TABLE 1 Enzyme activities in kidneys of Mpv17 -/- and Mpv17+/+ 7 - 9 months old mice

	Mpv17 -/-		Mpv17 +/+
	mmol/g of kidney wet weight		
GSH	2.19 ± 0.28 (93)[a]		2.35 ± 0.35
	U / mg of protein		
Superoxide Dismutase	17.1 ± 1.6 (102)		16.7 ± 0.8
Catalase	381 ± 25 (88)		431 ± 16
	nmol/min per mg of protein		
Glutathione Peroxidase	176 ± 12 (68)		259 ± 16
GSSG Reductase	98 ± 10 (85)		115 ± 10
Glutathione Transferase	508 ± 52 (98)		520 ± 36
γ - Glutamyl Transpeptidase	2,330 ± 295 (197)		1,180 ± 70

Data are given as means ± SEM (n= 10 animals)   [a] % of Mpv17+/+

Example 4: Mpv17 dependent activities of γ-glutamyl transp ptidase and glutathione peroxidase in fibroblasts

Changes of enzyme activities determined in kidneys of Mpv17-/- mice were similarly observed when in cultured Mpv17-/- (LUSVX) cells were compared to Mpv17 expressing (NIX15) cells (Table 2). Most prominently, in Mpv17-/- cells the activities of γ–GT were elevated by about six-fold, whereas the activities of GPx were lowered by one-third. The similarity in the change of γ–GT and GPx activity measured in Mpv17+/+ and Mpv17-/- kidney and fibroblast culture suggests that these alterations occur at the cellular rather than the organismal level. The following table summarizes the results:

TABLE 2   Enzyme activities in LUSVX and NIX15 fibroblasts

	LUSVX (Mpv17 negative)	NIX15 (Mpv17 expressing)
	U/ mg of protein	
Superoxide Dismutase (n=2)	10.7 (61)[a]	17.6
Catalase	410 ± 60 (87)	470 ± 90
	nmol/min per mg of protein	
Glutathione Peroxidase	53 ± 5 (66)	80 ± 7
γ-Glutamyl Transpeptidase	15 ± 0.4 (600)	2.5 ± 0.01

Data are given as means ± SEM (n=3-4)   [a] % of NIX15

Example 5: Mpv17 dependent changes in mRNA expression in fibroblasts

mRNA levels of the γ–GT and GPx genes were examined by quantitative RT-PCR in Mpv17expressing (NIX15) and Mpv17-/- (LUSVX) cells respectively (see Figure 2A below). γ–GT specific mRNA was enhanced by about 2-fold in Mpv17-/- cells. The expression of cellular GPx (cGPx), plasma GPx (pGPx), phospholipid hydroperoxide GPx (PHGPx) and of the nonselenium dependent GPx (nsGPx) was investigated. In Mpv17-/- cells only pGPx expression was decreased by about 80% (Figure 2A), which is basically consistent with the alteration in the activity of GPx (see Tables 1 and 2 above). Predominantly, pGPx appears to account for the overall low GPx activity. Remarkably, the expression of PHGPx, an enzyme responsible for protection from phospholipid peroxidation (R. L. Maser, B. S. Magenheimer and J. P. Calvet (1994), *Journal of Biological Chemistry*, **269**, pp. 27066-27073), was unaffected on the mRNA level.

The three different mouse SOD genes (CuZnSOD, MnSOD, ecSOD) show lower expression in Mpv17-/- cells, consistent with the lower SOD activity measured (see Table 2 above). No significant difference of xanthine dehydrogenase/ xanthine oxidase (XO) was detected on the mRNA level between Mpv17expressing and Mpv17 nonexpressing cells (see Figure 2A).

Example 6: Inhibition of γ-glutamyl transpeptidase activity restores glutathione peroxidase activity in Mpv17-/- cells

An inverse regulation of the glutathione-utilizing enzyme activities γ–GT and GPx was observed in Mpv17-/- animals and cells (see Tables 1 and 2 above). Because Mpv17-/- cells produce increased superoxide, a presumed regulatory function of the superoxide anion was tested by growing Mpv17-/- cells (LUSVX) in the presence of the SOD mimic MnTBAP (Y. Noda, M. Kohno, A. Mori and L. Packer (1999), *Journal Biological Chemistry*, **269**, pp. 23471-23476) Neither γ-GT nor GPx activities were changed significantly (Table 3) indicating that superoxide appears to have no role in the regulation of γ-GT and GPx in this system. Conversely, when Mpv17-/- cells were grown in the presence of acivicin, an efficient inhibitor of γ-GT, GPx activity was increased by about 1.6-fold. Thus, enzyme activities may be dependent on each

other in a way that γ–GT downregulates the activity of GPx. This inverse effect was also detected at the level of stable mRNA, as γ–GT inhibition led to a significant increase of pGPx and SOD mRNA levels (see Figure 2B).

TABLE 3   Activity of glutathione peroxidase and γ-glutamyl transpeptidase

in Mpv17 -/- cells (LUSVX) in presence of MnTBAP or acivicin

	LUSVX	LUSVX + MnTBAP	LUSVX	LUSVX + acivicin
		nmol/min per mg of protein		
Glutathione Peroxidase	25.0 ± 1.0	26.1 v 0.6 (104%) [a]	33.1 ± 1.3	53.5 ± 2.0 (162%)
γ-Glutamyl Transpeptidase	0.99 ± 0.02	0.94 ± 0.03 (95%)	0.96 ± 0.02	not detectable

Values are given as means ± SEM (n=3)   [a] % of controls

Example 7: Regulation of γ-GT and pGPx expression

The examples as documented herein above illustrate the following:

a) Reactive oxygen species in Mpv17 -/- cells

Using the ESR method superoxide was detected as the ROS species released from Mpv17 -/- fibroblasts. Production and secretion of superoxide are lower in Mpv17 expessing as compared to nonexpressing Mpv17-/- cells. These data are in line with the significance of ROS in the generation of glomerular injury (R. J. Johnson, D. Lovett, R. I. Lehrer, W. G. Couser and S. J. Klebanoff (1994), *Kidney International,*

45, pp. 352-359) and with an analysis of Mpv17-/- kidneys and isolated glomeruli, in which antioxidants were successfully used for therapeutic intervention in Mpv17-/- animals (C. J. Binder, H. Weiher, M. Exner and D. Kerjaschki (1999), *American Journal of Pathology*, **154**, pp. 1067-1075).

b) Activity and expression of oxidative enzymes

Activity and expression of enzymes involved in ROS and glutathione metabolism were determined in Mpv17-/- mice kidneys and fibroblasts in culture. An increase in γ-GT activity and a decrease in GPx activity were observed in both, Mpv17-/- kidneys and fibroblasts. In addition, a decrease in SOD activity was observed in Mpv17-/- fibroblasts. At the mRNA level, a negative correlation between the expression of γ-GT and the expression of the GPx and the SOD genes was observed in Mpv17-/- cells. All the three different SOD mRNAs tested were significantly decreased, but only the plasma GPx gene expression was strongly diminished, the latter presumably accounting for the decrease of GPx activity measured.

Negative correlations between the activity and expression of γ-GT and of enzymes involved in GSH and ROS metabolism have been reported earlier. Thus, inverse changes of GPx and γ-GT activities under the condition of oxidative stress were determined in rats exposed to cigarette smoke (C. V. Anand, U. Anand and R. Agarwal (1996), *Indian Journal Experimental Biology*, **34**, pp. 486-488) and in fetal mice exposed to alcohol (S. A. Amini, R. H. Dunstan, P. R. Dunkley and R. N. Murdoch (1996), *Free Radical Biology and Medicine*, **21**, pp. 357-365). Similarly, an inverse relationship of γ-GT and CuZnSOD expression has been noted recently in rat livers after iron poisoning (N. Taniguchi and Y. Ikeda (1998), *Advances in Enzymology and Related Areas of Molecular Biology*, **72**, pp. 239-278) But, in contrast to these previously described models, in the Mpv17 mouse model no chemical insult was applied.

c) Regulation of γ-GT and pGPx expression

In the absence of Mpv17 protein the γ–GT gene is upregulated while the mRNA level of pGPx is decreased. The mouse γ-GT gene is a single copy gene underlying intricate contol mechanisms involving at least seven promoters (N. Taniguchi and Y. Ikeda (1998), *Advances in Enzymology and Related Areas of Molecular Biology*, **72**, pp. 239-278). The membrane-bound γ–GT is involved in regulating cellular redox potential and intracellular GSH levels (T. C. Nichols, J. M. Guthridge, D. R. Karp, H. Molina, D. R. Fletcher and V. M. Holers (1998), *European Journal of Immunology*, **28**, pp. 4123-4129). The activity of γ–GT can be increased by glutathione depletion (R. J. van Klaveren, P. H. Hoet, J. L. Pype, M. Demedts and B. Nemery (1997), *Free Radical Biologoy and Medicine*, **22**, pp. 525-534) or by hyperoxia (A. Kugelman, H. A. Choy, R. Liu, M. M. Shi, E. Gozal and H. J. Forman (1994), *American Journal Respiratory Cell and Molecular Biology*, **11**, pp. 586-592) in different systems.

pGPx is an extracellular peroxidase of the selenium-containing GPx family, using GSH as well as and thioredoxin and glutaredoxin as thiol substrates (M. Björnstedt, J. Xue, W. Huang, B. Akesson and A. Holmgren (1994), *Journal of Biological Chemistry*, **269**, pp. 29382-29384). More abundant in kidney than in other tissues, pGPx is synthesized and secreted in the proximal tubules and in the glomeruli, consistent with its function in protecting kidney from extracellular oxidative damage (R. L. Maser, B. S. Magenheimer and J. P. Calvet (1994), *Journal of Biological Chemistry*, **326**, pp. 579-585; D. M. Tham, J. C. Whitin, K. K. Kim, S. X. Zhu and H. J. Cohen (1998), *American Journal of Physiology*, **275**, G1463-1471). Downregulation of pGPx as observed in Mpv17-/- cells weakens the protection against extracellular oxidative insult.

The γ–GT activity in Mpv17-/- cells controls the level of pGPx mRNA as γ–GT inhibition relieves this downregulation (Fig2b). This control might involve imbalanced levels of intra- or extracellular GSH or superoxide due to enhanced γ–GT activity, presumably mediated by the activation of superoxide responsive transcription factors such as NF-κB or AP-1 (H. L. Pahl and P. A. Baeuerle (1994), *Bioessays*, **16**, pp.

497-502). However, superoxide removal does neither affect the γ-GT nor the GPx activity, arguing against superoxide as a regulator.

Several genes relevant to the development of the disease phenotype, i.e. MMP-2 and its regulator TIMP-2, have been shown to be upregulated in Mpv17-/- mice earlier (A. Reuter, A. Nestl, R. M. Zwacka, J. Tuckerman, R. Waldherr, E. M. Wagner, M. Hoyhtya. A. M. Meyer zum Gottesberge, P. Angel and H. Weiher (1998), *Molecular Biology of the Cell*, **9**, pp. 1675-1682). Since antioxidant intervention is effective in phenotype prevention in our model (C. J. Binder, H. Weiher, M. Exner and D. Kerjaschki (1999), *American Journal of Pathology*, **154**, pp. 1067-1075), these alterations should be consequences rather than causes to ROS generation. By contrast, the data presented here suggest that overproduction of γ-GT in these animals is causal to elevated ROS levels (see below).

d) Origin of enhanced ROS levels in Mpv17 -/- mice

Enzymes most affected in Mpv17-/- kidneys and cells, γ-GT and pGPx, both exert their enzymatic activity predominantly in the extracellular space. In particular, γ-GT expression and activity are enhanced in the absence of the Mpv17 function. Cells overproducing γ-GT should be efficiently protected against intracellular oxidative injury by increased supply of intracellular GSH. Extracellular GSH is metabolised by γ-GT to glutamate and cysteinylglycine which in contrast to GSH can directly enter cells and thus provide them with a source of cysteine (M. W. Lieberman, A. L. Wiseman, Z. Z. Shi, B. Z. Carter, R. Barrios, C. N. Ou, P. Chevez-Barrios, Y. Wang, G. M. Habib, J. C. Goodman, S. L. Huang, R. M. Lebovitz and M. M. Matzuk (1996), *Proceedings of the National Academy of Sciences of the U.S.A.*, **76**, pp. 5606-5610). The latter is present at lowest concentration of all amino acids and a limiting component for intracellular de novo GSH synthesis. Thus, γ-GT, localized at the luminal surface of the renal proximal tubules, plays a key role in cysteine and glutathione homeostasis in maintaining cellular GSH levels (A. Kugelman, H. A. Choy, R. Liu, M. N. Shi, E. Gozal and H. J. Forman (1994), *American Journal Respiratory Cell and Molecular Biology*, **11**, pp. 586-592).

At the same time, increased γ-GT activity might lead to a depletion of extracellular GSH and thereby weaken the resistance against extracellular ROS. In mice, however, this is unlikely, because plasma GSH levels are about 100-fold higher than in humans (O. W. Griffith and A. Meister (1979), *Prodeedings of the National Academy of Sciences of the U.S.A.*, **76**, pp. 5606-5610), that is well above a critical substrate concentration for pGPx activity of <0.5 μM (A. Wendel and P. Cikryt (1980), *FEBS Letters*, **120**, pp. 209-211). Instead, increased γ-GT activity may directly enhance superoxide in the Mpv17-/- system. Such direct production of superoxide by γ-GT activity was recently demonstrated in an in vitro system containing GSH and transferrin as an iron source. It was shown that superoxide was generated by the reaction of the GSH breakdown product cysteinylglycine (R. Drozdz, C. Parmentier, H. Hachad, P. Leroy, G. Siest and M. Wellman (1998), *Free Radicals Biology and Medicine*, **25**, pp. 786-792). Superoxide can instantly undergo a Fenton type reaction to turn into the highly noxic hydroxyl radical, causing lipid- and protein peroxidation (R. Drozdz, C. Parmentier, H. Hachad, P. Leroy, G. Siest and M. Wellman (1998), *Free Radicals Biology and Medicine*, **25**, pp. 786-792). In vivo, hydroxyl radical generation and lipid peroxidation in the presence of metals and under conditions of enhanced γ-GT activity have been described earlier in rat liver (K. E. Brown, M. T. Kinter, T. D. Oberley and D. R. Spitz (1998), *Free Radical Biology and Medicine*, **24**, pp. 545-555; A. A. Stark, E. Zeiger, D. A. Pagano (1993) *Carcinogenesis*, **14**, pp. 183-189; A. Paolicchi, R. Tongiani, P. Tonarelli, M. Comporti and A. Pompella (1997), *Free Radicals Biology and Medicine*, **22**, pp. 853-860).

The above described experiments clearly demonstrate that γ-GT activity plays a key role in the generation of extracellular ROS. Recently, γ-GT upregulation has been reported to be causal to oxidation damage during short-term ischemia of rat kidney and this effect was inhibitable by acivicin (J. C. Cutrin, B. Zingaro, S. Camandola, A. Boveris, A. Pompella and G. Poli (2000), *Kidney International*, **57**, pp. 526-533). Thus, the use of γ-GT inhibitors provides a potent and useful treatment of ROS degenerated diseases and injuries in humans as well.

Mpv 17-/-mice, i.e. mice of the glomerulosclerosis reference strains are treated, in accordance with this invention, by oral administration of 5 to 50 mg/kg activicin (AT-125) for several weeks. The protective use of activicin is analyzed by pathological methods and/or molecular means.